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Muscodor cinnamomi, a new endophytic species from *Cinnamomum bejolghota*

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Abstract — *Muscodor cinnamomi* is described as a new species, endophytic within leaf tissues of *Cinnamomum bejolghota* (*Lauraceae*) in Doi Suthep-Pui National Park, Northern Thailand. Molecular analysis indicated differences from the five previously described *Muscodor* spp. Volatile organic compounds analysis showed that *M. cinnamomi* produced azulene (differentiating it from *M. crispans*) but did not produce naphthalene (differentiating it from *M. albus, M. roseus,* and *M. vitigenus*).

Key words — sterile ascomycete, cinnamon, endophytes, volatile compounds

Introduction

Plants are reservoirs of untold numbers of endophytic organisms (Bacon & White 2000). By definition, these microorganisms (mostly fungi and bacteria) reside in the tissues beneath the epidermal cell layer and cause no apparent harm to the host (Azevedo et al. 2000, Hyde & Soytong 2008). Endophytes from rainforest and medicinal plants have been studied for their volatile antibiotic and other medicinal characteristics (Strobel et al. 2003, Huang et al. 2008, 2009, Mitchell et al. 2008, Tejesvi et al. 2009, Aly et al. 2010). Five endophytes characterized by sterile mycelium that have recently been described as novel fungi are *Muscodor albus* isolated from *Cinnamomum zeylanicum* (*Lauraceae*) in Honduras (Worapong et al. 2001), *M. roseus* from *Grevillea pteridifolia* (*Proteaceae*) in the Northern Territory of Australia (Worapong et al. 2002), *M. vitigenus* from *Paullinia paullinioides* (*Sapindaceae*) in Lake Sandoval (Daisy et al. 2002), *M. crispans* from *Ananas ananassoides* (*Bromeliaceae*) in the Bolivian Amazon (Mitchell et al. 2008), and *M. yucatanensis* from *Bursera*

simaruba (*Burseraceae*) in the Northeastern Yucatan Peninsula of Mexico (González et al. 2009). All *Muscodor* species grow slowly, have felt-like mycelia, and produce a distinctive odor. Gas chromatography and mass spectrometry (GC/MS) can be used to identify *Muscodor* species based on differences in the volatile compounds that they produce (Strobel et al. 2001).

In the present study an endophyte (CMU-Cib 461) was recovered from leaf tissue of a wild cinnamon tree (*Cinnamomum bejolghota*) growing in Doi Suthep-Pui National Park, Thailand. The strain produced a mixture of volatile compounds including propanoic acid and alcohol; these have antagonistic activities and can be used to identify the particular *Muscodor* species. CMU-Cib 461 possesses cultural, chemical, and molecular characteristics that differ from *M. albus*, *M. crispans*, *M. roseus*, *M. vitigenus,* and *M. yucatanensis.* We conclude that CMU-Cib 461, based on its unique features, represents a new species of *Muscodor*, for which we propose the name *Muscodor cinnamomi*.

Materials and methods

Fungal isolation

Ten healthy leaves of *Cinnamomum bejolghota* were collected from plants growing in Doi Suthep-Pui National Park, Northern Thailand (alt. 950 m) during May 2008. Totally, 250 tissue squares (5 × 5 mm) were cut from the leaf samples. All leaf tissues squares were surface sterilized in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min and 95% ethanol for 30 s under a laminar flow hood (Nuangmek et al. 2008). The sterilized samples were placed in Petri dishes containing 2% malt extract agar, 0.05% streptomycin sulfate and 0.03% rose bengal (Bussaban et al. 2001). Petri dishes were sealed with Parafilm and incubated at room temperature (25±2°C) for one week. The fungi growing out from the samples were aseptically transferred to two culture media, potato dextrose agar (PDA) and malt extract agar (MA); pure isolates were maintained in corn meal agar (CMA) slants. Various methods were tried to stimulate spore production (Guo et al. 1998).

Scanning electron microscopy

Scanning electron microscopy was preformed on isolate CMU-Cib 461 following procedures described by Castillo et al. (2005). A piece of agar with fungus was placed in a filter paper packet and then placed in 2% glutaraldehyde vapor, a wetting agent, and aspirated over night. Samples were then dehydrated in an ethanol series (15 mins at 5, 10, 15, 20, 40, 50, 70, 80, 95 and 100%) and in an acetone series (10 mins at 10, 15, 20, 40, 50, 70, 80, 95 and 100%). The fungal material was critically point dried, gold sputter coated, and images observed under a JEOL JSM-5910LV SEM using a high vacuum mode.

Qualitative analysis of CMU-Cib 461 volatiles

CMU-Cib 461 was grown in 5 ml Aglelent® clear glass vials containing PDA for 10 days at room temperature (25±2°C). Volatile compounds produced by the fungus were analyzed on an automatic Agilent Technologis GC 7890 gas chromatograph column

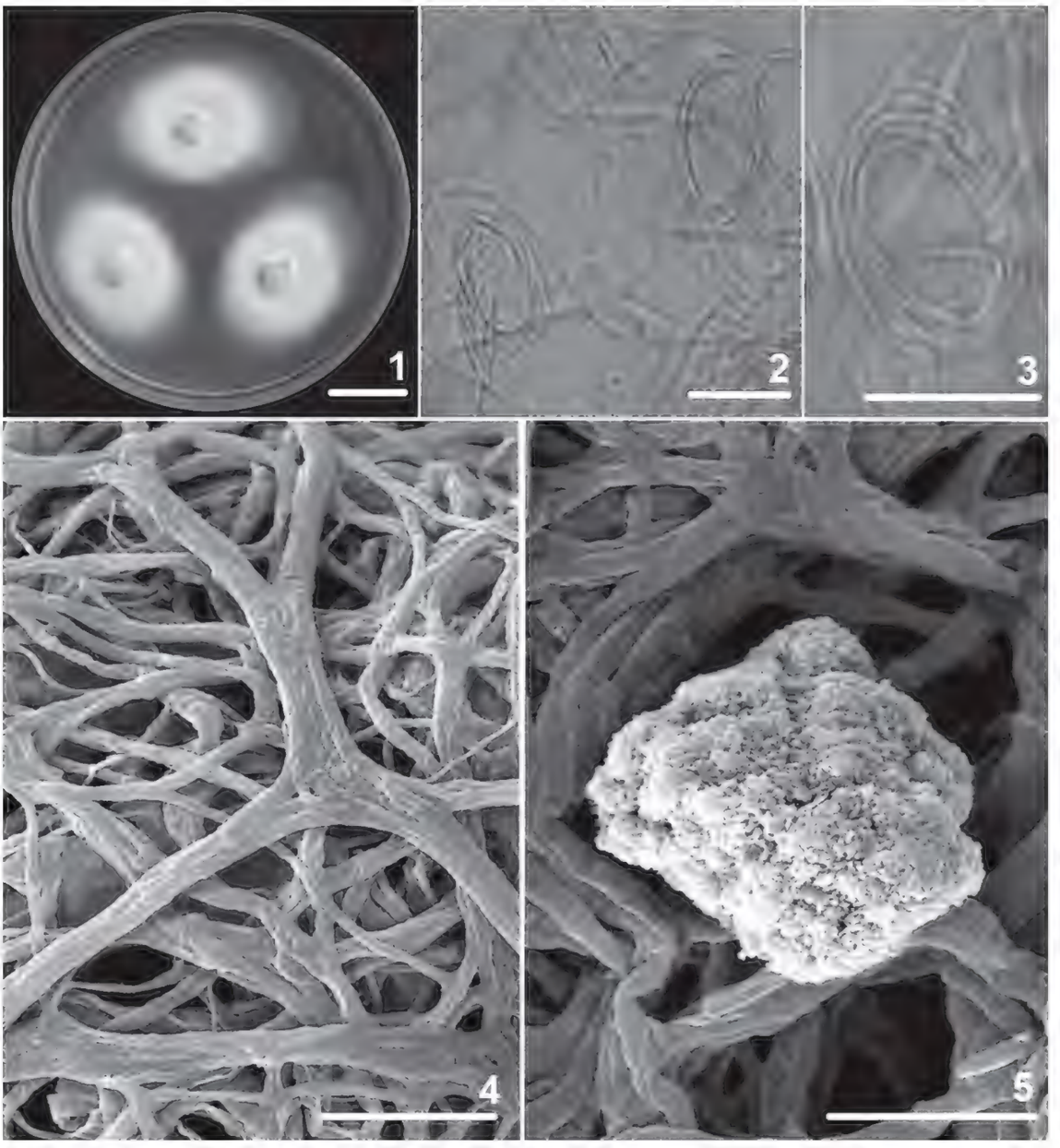
containing a HP-5MS 30 m × 0.25 mm I.D. × 0.25 μm. The column was temperature programmed as follows: 32°C for 2 min followed to 220°C at a rate of 5°C/min. The carrier gas was ultra high purity helium released at a rate of 1.5 mL/min. Prior to trapping the volatiles, the fiber was conditioned at 250°C for 39.6 min under a flow of helium gas. The gas chromatograph was interfaced to a MSD 5973 (EI) mass selective detector (mass spectrometer) operating at unit resolution. Acquisition and processing data were performed on the MSD 5973 (EI) software system. Initial identification of the volatile compounds produced by CMU-Cib 461 was made through library comparison using the NIST database, and compared with the original isolates, *M. albus* strain 620 (Strobel 2006) and strain E-6 (Strobel et al. 2007).

Fungal cultures and DNA extraction

Genomic DNA was extracted by a modified SDS-CTAB method (Bussaban et al. 2005). Strain CMU-Cib 461, isolated from *C. bejolghota* leaves, was subcultured onto PDA and incubated for 10 days. Mycelium was harvested, freeze dried, and ground into a fine powder with a pestle and mortar. About 15 mg of powdered mycelium was suspended in 1 mL of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4, 20 mg/mL proteinase K), transferred into 1.5 mL Eppendorf tube and kept at 4°C to prevent endonuclease activity during rehydration of the sample. SDS was added to a final concentration of 2%, vortexed and incubated 30 min at 65°C. After centrifugation for 15 min at 14,000 rpm, the supernatant was transferred to a new sterile 1.5 mL Eppendorf tube. The volume of supernatant was measured and the NaCl concentration was adjusted to 1.4 M, and one-tenth volume of 10% CTAB buffer (10% CTAB, 500 mM Tris-HCl, 100 mM EDTA, pH 8.0) was added. The solution was thoroughly mixed and incubated for 10 min at 65°C. After cooling for 2 min at 15°C, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added, thoroughly mixed and the tube was centrifuged 15 min at 14,000 rpm. The extraction was repeated until the interface was clear. The supernatant was removed to a new Eppendorf tube, containing 2 volumes of cold 100% ethanol. After DNA precipitation, the pellet was centrifuged for 15 min at 14,000 rpm and 4°C. The pellet was washed with 70% ethanol and dried at room temperature. It was resuspended in 100 mL of 0.002% RNase (5 mg/mL) in TE buffer and incubated for 1 h at 37°C. The suspension was stored at -20°C pending use for PCR amplification.

Fungal ITS regions sequencing and phylogenetic analysis

The internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA were separately amplified in a 25 mL reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems) under these reaction conditions: 1 mL of template DNA extraction, 0.2 mM dNTP, 0.2 mL of FastTaq (Applied Biosystems), 0.2 mM each of primers, 2.5 mL of the supplied 103 PCR buffer with $MgCl_2$, and sterile water to bring the volume to 25 mL. The ITS regions were amplified by using ITS4 and ITS5 primers. Amplification of ITS regions was for 30 cycles (initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min). PCR products were analyzed by electrophoresis in 1% agarose gels in TAE buffer (20 mM Tris-Acetate, 1 mM EDTA, pH 8.0) and viewed by staining with ethidium bromide. PCR products were purified using PCR clean up Gel extraction



Figs. 1–5. *Muscodor cinnamomi* 1. A culture of *Muscodor cinnamomi* CMU-Cib 461 growing on PDA, bar = 2 cm. 2–3. Light microscope micrographs of coiling formation of fungal hyphae, bars = 5 μm. 4–5. Scanning electron micrographs. 4. Hyphal cells from the colony edge showing fused, rope-like hyphal cells, bar = 10 μm. 5. Fused hyphal cells and a cauliflower-like structure, bar = 5 μm.

NucleoSpin® Extract II purification Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed and sequences determined automatically in a genetic analyzer (1ST Base, Malaysia) using the PCR primers mentioned above. Sequences obtained in this study were compared to those from GenBank database using the BLAST software on the NCBI website: (http://www.ncbi.nlm.nih.gov/BLAST/). After multiple alignment of selected sequencher with Clustal X. Phylogenetic trees were constructed using the PUAP beta 10 software version 4.0 (Swofford 2002).

Results

Taxonomic description

Muscodor cinnamomi Suwannarach, K.D. Hyde & Lumyong, **sp. nov.** Figs 1–5

MycoBank # MB518008, GenBank # GQ848369

Fungus in natura cum Cinnamomi bejolghota *consociatus et est deuteromycete myceliis sterilibus pertinens. Coloniae fungales est luteus in vitro examinati in loco cum sol lux. Sporae velcorpora fructificantia substatibus ullis non observata. Hyphae (0.9–5.2 µm) vulgo ramificantes et convolventes, fila stripformia et spiras perfectas (4.5–12 µm) formantes. In vitro examiniter corpores colifloriform (6.3–14 µm) e repletus forma hyphae.*

Etymology: *cinnamomi*, from the name of the host plant.

Holotype: Thailand, Doi Suthep-Pui National Park; from a leaf of *Cinnamomum bejolghota* (*Lauraceae*), May 2008, Nakarin Suwannarach; **holotype** – dried culture, SDBR CMU-Cib 461. (Living culture, BCC38842).

Teleomorph: Unknown.

In nature, the fungus is associated with *Cinnamomum bejolghota* and it is an ascomycete with sterile mycelium. Fungal colonies whitish on all media (PDA, MA and CMA) when grown in darkness (Fig. 1), pale orange when grown in natural light. Hyphae (0.9–5.2 µm thick) commonly appearing as fused rope-like strands, branching (Fig. 4); with coils (4.5–12 µm diam.; Figs 2, 3) and cauliflower-like bodies (6.3–14 µm; Fig. 5). Mycelium on PDA reaching 9 cm in 2–3 weeks and producing a fruity odor. Spores and other fruiting bodies did not develop under any conditions tested.

Molecular phylogeny of *Muscodor cinnamomi* CMU-Cib 461

Partial ITS1 5.8 ITS2 rDNA sequences of *M. cinnamomi* were obtained and compared with GenBank database. After searching the ITS-5.8S rDNA sequences, 635 bp of *M. cinnamomi* (GQ848369) was subjected to an advanced BLAST search. The ITS1 5.8S ITS2 rDNA sequences of *M. cinnamomi* blasted five type strains of *Muscodor* species. The result showed that there was a 99, 99, 99, 98 and 90% similarity with *M. albus* (AF324336), *M. roseus* (AY034665), *M. crispans* (EU195297), *M. vitigenus* (AY100022) and *M. yucatanensis* (FJ917287), respectively.

Parsimony analysis of the alignment yielded 100 most parsimonious trees with total length of 873 steps (CI = 0.705, RI = 0.746, RC = 0.526, HI = 0.294), one of which is shown in Fig. 6. *Muscodor cinnamomi* and *Muscodor* species from GenBank formed a monophyletic clade (clade I) with a high bootstrap support (99%), and formed a sister group to *Anthostomella* (clade II) with 83% bootstrap support. *Muscodor* species are more closely related to the *Xylariaceae* than *Amphisphaeriaceae* with 100% bootstrap support.

Volatile compounds from *M. cinnamomi* (CMU-Cib 461)

Muscodor cinnamomi (CMU-Cib 461) produced at least 11 volatile compounds. These could be positively identified on the basis of a GC/MS

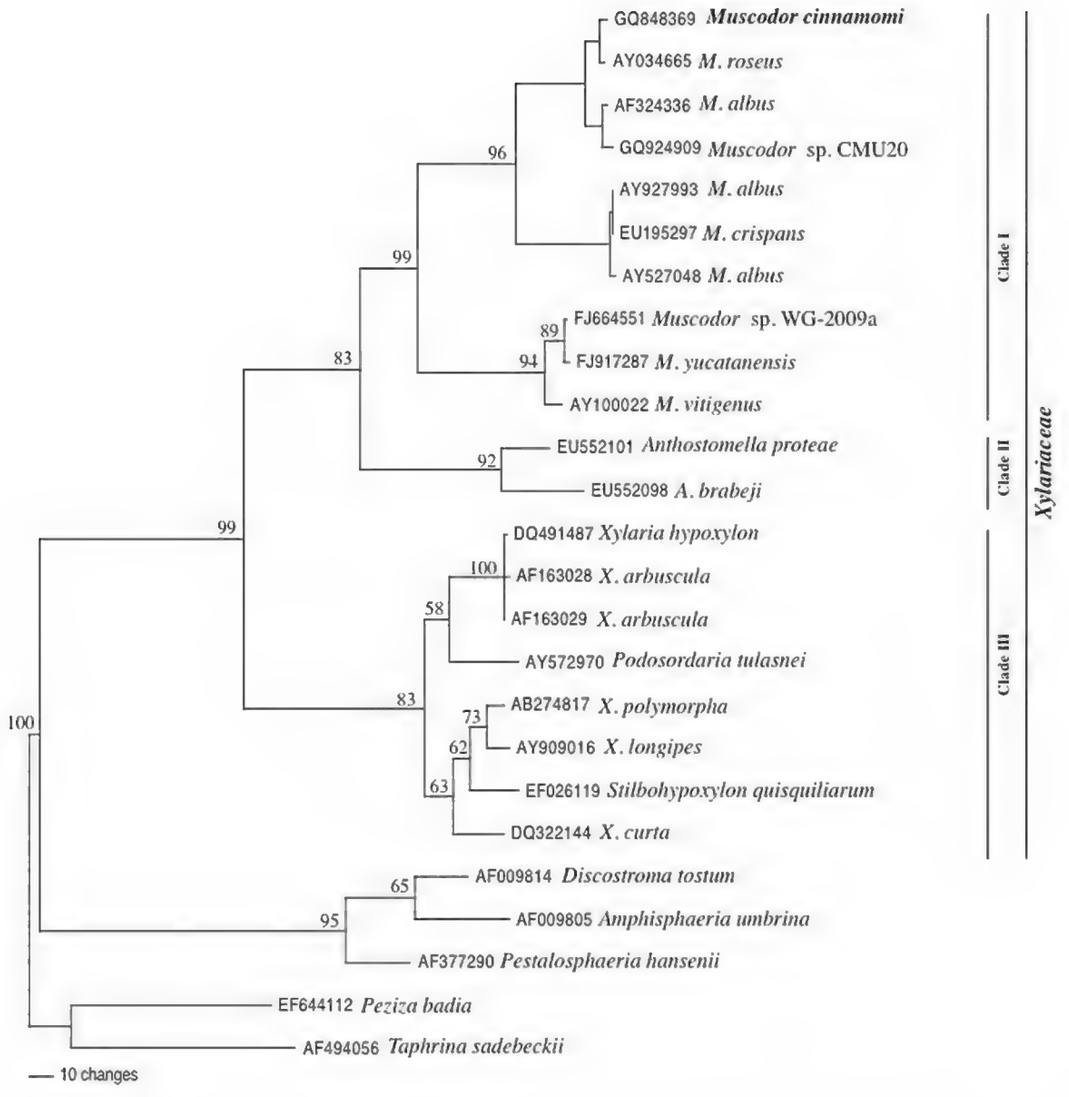


Fig 6. One of 100 most parsimonious trees inferred from a heuristic search of the ITS1-5.8S-ITS2 rDNA sequence alignment of 25 isolates of *Muscodor* and related genera. *Peziza badia* and *Taphrina sadebeckii* were used to root the tree. The size of the branches is indicated with a scale bar. Branches with bootstrap values ≥ 50% are shown at each branch.

comparison with authentic standards obtained from commercial sources as well as organic synthesis. The compounds were identified primarily on the basis of their mass spectral properties when compared to the NIST database. Of the compounds produced by this organism the most abundant were propanoic acid, 2-methyl, methyl ester, butanoic acid, 2-methyl, methyl ester and cis-2,4-dimethylthiane,S,S-dioxide with total area higher than 10% (Table 1). A number of other volatiles appeared that were unique to this isolate, including cis-2,4-dimethylthiane,S,S-dioxide; β-humolene; cyclopentane; eudesma4(14),11-diene and 1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane compounds. In addition, the fungus produced azulene, but no naphthalene compounds.

Table 1. GC/MS analysis of the volatile compounds produced by *Muscodor cinnamomi* (CMU-Cib 461) culture in 5.0 mL clear glass vial Aglelent® for 10 days.

RT (min:s)	Total area (%)	Analysis compound	*M/z*
3:15	1.10	(S)-(+)-5-methyl-1-heptanol	130
3:32	5.49	ethyl acetate	88
4:35	32.26	propanoic acid,2-methyl,methyl ester	102
5:38	11.35	cis-2,4-dimethylthiane,S,S-dioxide*	162
5:41	7.69	cyclopentane*	70
6:38	14.90	butanoic acid,2-methyl,methyl ester	116
9:29	3.12	1-butanol,3-methyl,acetate	130
27:42	3.23	β-humulene*	204
30:89	8.58	azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.α., 7.α., 8a.β)]	204
30:90	7.32	Eudosma-4(14),11-diene*	107
34:48	2.66	1,1,1,5,7,7,7-heptamethyl-3,3-bis (trimethylsiloxy) tetrasiloxane	444

Abbreviations: * = compounds found in *M. cinnamomi* but not in other *Muscodor* species; RT = retention time; *M/z* = mass to charge ratio.

Discussion

Muscodor cinnamomi is introduced as a new species based on differences in colony characteristics, growth rate, ITS sequence data and volatile compounds produced. *Muscodor cinnamomi* (CMU-Cib 461) produced a white mycelium on a PDA. Spores or fruiting structures did not develop on any media including ones containing the host plant material, cinnamon leaves. In this respect it is similar to other *Muscodor* species. The hyphae tend to intertwine to form rope-like strands. Other species of *Muscodor* also have this tendency (Worapong et al. 2001, 2002). The fungus also produces cauliflower-like structures, which is similar to *M. crispans*. The features of *M. cinnamomi* (CMU-Cib 461) are similar to *M. albus, M. crispans, M. vitigenus* and *M. yucatanensis* which produce whitish mycelium on all media tested in artificial light (Worapong et al. 2001, Daisy et al. 2002, Mitchell et al. 2008, González et al. 2009). *Muscodor cinnamomi* developed a pale orange coloured mycelium in natural light, while *M. crispans* produces a pale pink mycelium in natural light (Mitchell et al. 2008). Phylogenetic analysis of the sequences of ITS1, 5.8S, and ITS2 showed that *M. cinnamomi* was closely related the other *Muscodor* species, which are related to family *Xylariaceae* (Worapong et al. 2001, 2002).

When measured by GC/MS, the fungus consistently produced alcohols, esters and small molecular weight acids, in the gas phase, when grown on PDA. *Muscodor cinnamomi* produces propanoic acid,2-methyl,methyl ester, which is similar to other *Muscodor* species. However, there are differences in other

Table 2. Synopsis of azulene and naphthalene production* by *Muscodor* species.

Species	Azulene	Naphthalene	Data source
M. albus	+	+	Worapong et al. 2001
M. cinnamomi	+	–	This paper
M. crispans	–	–	Mitchell et al. 2008
M. roseus	+	+	Worapong et al. 2002
M. vitigenus	+	+	Daisy et al. 2002
M. yucatanensis	n	–	González et al. 2009

* (+) = production; (–) = non-production; (n) = unreported.

compounds produced by the different *Muscodor* species (Table 2). The volatile compounds showed inhibition ability and lethal activity against a number of plant and human pathogens (Strobel et al. 2001, Worapong & Strobel 2009). Details on the bioactivities of this interesting genus appear elsewhere (Worapong et al. 2001, 2002, Daisy et al. 2002, Ezra et al. 2004, Strobel 2006, Strobel et al. 2007, Mitchell et al. 2008). The strain CMU-Cib 461 shared all of the common features of previously described *Muscodor* species but there were a number of different aspects to the taxon that distinguished it from other *Muscodor* species.

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Literature cited

Aly AH, Debbab A, Kjer A, Proksch P. 2010. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers (In press).

Azevedo JL, Maccheroni W Jr, Pereira JO, Araujo WL. 2000. Endophytic microorganisms: a review on insect control a recent advances on tropical plant. Electron J Biotechnol 3: 40–65. doi:10.2225/vol3-issue1-fulltext-4

Bacon CW, White JF. 2000. Microbial Endophytes. Marcel Dekker, New York.

Bussaban B, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD. 2001. Endophytic fungi from *Amomum siamense*. Can J Microbiol 47: 943–948. doi:10.1139/cjm-47-10-943

Bussaban B, Lumyong S, Lumyong P, Seelanan T, Park DC, McKenzie EHC, Hyde KD. 2005. Molecular and morphological characterization of *Pyricularia* and allied genera. Mycologia 97: 1002–1011. doi:10.3852/mycologia.97.5.1002

Castillo U, Myers S, Browne L, Strobel G, Hess WM, Hanks J, Reay D. 2005. Scanning electron microscopy of some endophytic streptomycetes in snake vine–*Kennedia nigricans*. Scanning 27: 305–311.

Daisy B, Strobel G, Ezra D, Castillo U, Bairn G, Hess WM. 2002 *Muscodor vitigenus* anam. sp. nov., an endophyte from *Paullinia paullinioides*. Mycotaxon 81: 463–475.

Ezra D, Hess WM, Strobel GA. 2004. New endophytic isolates of *Muscodor albus*, a volatile antibiotic producing fungus. Microbiology 150: 4023–4031. doi: 0.1099/mic.0.27334-0

González MC, Anaya AL, Glenn AE, Macías-Rubalcava ML, Hernández-Bautista BE, Hanlin RT. 2009. *Muscodor yucatanensis* a new endophytic ascomycete from Mexican chakah, *Bursera simaruba*. Mycotaxon. 110: 363–372.

Guo LD, Hyde KD, Liew ECY. 1998. A method to promote sporulation in palm endophytic fungi. Fungal Divers 1: 109–113.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33: 61–75.

Huang WY, Cai YZ, Surveswaran S, Hyde KD, Corke H, Sun M. 2009. Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species. Fungal Divers 36: 69–88.

Hyde KD, Soytong K. 2008. The fungal endophyte dilemma. Fungal Divers 33: 163–173.

Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D. 2008. *Muscodor crispans*, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. Fungal Divers 31: 37–43.

Nuangmek W, McKenzie EHC, Lumyong S. 2008. Endophytic fungi from wild banana (*Musa acuminata* Colla) works against anthracnose disease caused by *Colletotrichum musae*. Res J Microbiol 3: 368–374.

Strobel GA. 2006. *Muscodor albus* and its biological promise. J Ind Microbiol Biotechnol 33: 514–522. doi:10.1007/s10295-006-0090-7

Strobel GA, Dirske E, Sears J, Markworth C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology 147: 2943–2950. doi: 10.1099/mic.0.27334-0

Strobel GA, Kluck K, Hess WM, Sears J, Ezra D, Vargas PN. 2007. *Muscodor albus* E-6, an endophytic of *Guazuma ulmifolia* making volatile antibiotics: isolation, characterization and experimental establishment in the host plant. Microbiology 153: 2613–2620. doi: 0.1099/mic.0.2007/008912-0

Swofford DL. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), beta version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Tejesvi MV, Tamhankar SA, Kini KR, Rao VS, Prakash HS. 2009. Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmaceutically important medicinal trees. Fungal Divers 38: 167–183.

Worapong J, Strobel GA. 2009. Biocontrol of root rot of kale by *Muscodor albus* strain MFC2. Biocontrol 54: 301–306. doi:10.1007/s10526-008-9175-8

Worapong J, Strobel GA, Daisy BH, Castillo U, Baird G, Hess WM. 2002. *Muscodor roseus* sp. nov., an endophyte from *Grevillea pteridifolia*. Mycotaxon 81: 463–475.

Worapong J, Strobel GA, Ford E, Li JY, Baird G, Hess WM. 2001. *Muscodor albus* anam. gen. et sp. nov., an endophyte from *Cinnamomum zeylanicum*. Mycotaxon 79: 67–79.